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Review

Advanced microscopy techniques for quantitative analysis in neuromorphology and neuropathology research: current status and requirements for the future

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ABSTRACT

Visualizing neuromorphology and in particular neuropathology has been the focus of many researchers in the quest to solve the numerous questions that are still remaining related to several neurological and neuropsychiatric diseases. Over the last years, intense research into microscopy techniques has resulted in the development of various new types of microscopes, software imaging systems, and analysis programs. This review briefly discusses some key techniques, such as confocal stereology and automated neuron tracing and reconstruction, and their applications in neuroscience research. Special emphasis is placed on needs for further developments, such as the demand for higher-throughput analyses in quantitative neuromorphology. These developments will advance basic neuroscience research as well as pharmaceutical and biotechnology research and development.

Contents

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1. Introduction

In neuromorphology and neuropathology, quantitative analyses are the primary factor towards advanced understanding of normal and pathological composition of neural tissue. Design-based stereology has become widely accepted in analyzing populations of three-dimensional (3D) structures such as neurons, while neuron tracing and reconstruction are utilized for morphological analyses of single neurons or networks of neurons. Many new insights into disease pathogenesis were gained through quantification of profound or, in particular, subtle changes in regional volumes of brain regions, numbers of neurons and glial cells, size of cells, neuron arborization, spine numbers, etc. Although new techniques have evolved during recent years, efficiency and quality of these techniques can still be improved.

In this review we first briefly explain the meaning of design-based stereology, neuron tracing and reconstruction. Subsequently we focus on past, present, and possible future developments in the aforementioned disciplines. Moreover, we give examples how researchers apply these techniques in neuroscience, and finally outline some needs for the future in order to perform higher-throughput analyses.

2. Design-based stereology

Design-based stereology is a discipline to perform precise quantitative evaluations of the structure of small and large 3D objects, hence the name stereology (stereos = solid; logos = knowledge) (Weibel, 1979; Howard, 1998; Schmitz and Hof, 2005; Glaser et al., 2007).

By analyzing systematically and randomly sampled two-dimensional (2D) sections or 3D subsamples of tissue, one can calculate morphometric parameters of a 3D structure, while eliminating almost all potential methodological bias. In recent years, design-based stereology has become the gold standard for quantitative analyses of 3D structures at the histological level in biomedical research.

2.1. Confocal microscopy

Confocal microscopy was patented in 1957 by Marvin Minsky (Kubinova et al., 2004), and is increasingly being used in biomedical research because it enables 3D analysis of thick microscopic specimens such as those encountered in studies involving living cells and tissues. The term ‘confocal’ means that only the light from one single focal plane is used to create the image of that specific focal plane. Accordingly, one can look at a specimen using one confocal plane at a time, a feature known as optical sectioning. In non-confocal microscopy, also called wide-field microscopy, a wide focal spread of the specimen is homogenously illuminated, and optical sectioning is not possible because of the additional background signal from out-of-focus structures above and below the focal plane. This kind of light scattering results in lower image quality, resolution, and contrast (Denk and Svoboda, 1997; Rigby and Goldie, 1999; Saggau, 2006). The use of confocal microscopes eliminates all out-of-focus light from other focal planes, and, thus, creates sharper images with better signal-to-noise ratio and better lateral (X,Y) and vertical/axial (Z) resolution (see, e.g., Conchello and Lichtman, 2005). As a result, thicker specimens can be used for imaging. By focusing through a thick specimen and taking an image on each focal plane with fixed distances in between, one can obtain image stacks of thin serial optical sections (Z-series or image stacks). These digital image stacks can then be used to perform quantitative analyses according to the principles of design-based stereology, or to perform 3D reconstructions to assess the organization of certain structures within the specimen (Pawley, 1996; Rigby and Goldie, 1999).

Several new modalities of optical sectioning microscopy have been developed that all use the confocal principle, such as laser/spot/point scanning confocal microscopy, spinning disk microscopy, two-photon or multi-photon microscopy. All of these techniques have their specific advantages and drawbacks, and the choice of technique depends on the specific application in the performed research.

In confocal laser scanning microscopy (CLSM), a pinhole is located in the image pathway and aligned with the illumination point. This aperture makes sure that all of the fluorescent light coming from out-of-focus locations within the inspected tissue is obstructed. The main disadvantage of eliminating the unwanted out-of-focus light is the need to increase the illumination strength of the light source, which can cause photobleaching and photodamage (Denk and Svoboda, 1997; Conchello and Lichtman, 2005; Saggau, 2006). A more recent and advanced technique becoming widely adopted is two-photon microscopy, which was invented almost 20 years ago (Denk et al., 1990). Multi-photon microscopy is an extension of two-photon microscopy, since it uses more than two photons to excite fluorescence, resulting in an even tighter excitation volume and less useless obsolete image information than the two-photon variant (Saggau, 2006). In two-photon and multi-photon microscopy, only the fluorophores at a particular focal plane become excited, resulting in lack of out-of-focus excitation and less photobleaching compared to CLSM (Denk and Svoboda, 1997; Svoboda et al., 1997; Centonze and White, 1998; König, 2000). A drawback of both CLSM and two-photon microscopy is the fact that they work with point illumination (Saggau, 2006; Svoboda and Yasuda, 2006). Point illumination means that the specimen is scanned one point at a time (pixel by pixel and line by line), which limits the speed of scanning.

While CLSM and two-photon microscopy are based on time-consuming single-point scanning, spinning disk systems use multipoint or slit scanning methods that collect emission photons from multiple spots at a time. This process saves time without compromising the spatial resolution (Lucitti and Dickinson, 2006). Confocal spinning disk microscopy (CSDM) is extremely useful for applications involving real-time fluorescent imaging of living cells, since the capturing of images by digital cameras is fast, and CSDM produces only minimal photodamage and photobleaching (Wang et al., 2005). The principle of the confocal effect of CSDM is as follows: the spinning disk splits the light beam up in multiple beamlets that scan the specimen. In other words, rather than single points, a raster of multiple points are scanned, resulting in faster imaging and reduced photobleaching because the excitation energy is split into multiple beams scanning all points (Inoue and Inoue, 2002; Wang et al., 2005). The confocal effect is created when the emission light runs back through the spinning disk (Toomre and Pawley, 1995). Another advantage of CSDM is the fact that in these systems, it is possible to use conventional mercury lamps with a broad range of wavelengths, instead of single wavelength laser light sources. With mercury lamps, the desired wavelength is obtained with normal excitation and emission filter cubes (Saito et al., 2008).

2.2. Some examples of applications of confocal microscopy in neuromorphology and neuropathology research

Although traditional wide-field microscopy is useful for many applications in neuroscience, certain neuronal structures and processes cannot be visualized with this conventional way of microscopy. The use of confocal microscopy is a prerequisite for imaging cellular and subcellular objects. Here we give some